SPECIAL GUEST EDITOR SECTION: FOOD ALLERGENS NEW METHODS

Target Selection Strategies for LC-MS/MS Food Allergen Methods

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The detection and quantitation of allergens as contaminants in foods using MS is challenging largely due to the requirement to detect proteins in complex, mixed, and often processed matrixes. Such methods necessarily rely on the use of proteotypic peptides as indicators of the presence and amount of allergenic foods. These peptides should represent the allergenic food in question in such a way that their use is both sensitive (no false-negatives) and specific (no false-positives). Choosing such peptides to represent food allergens is beset with issues, including, but not limited to, separated ingredients (e.g., casein and whey), extraction difficulties (particularly from thermally processed foods), and incomplete sequence information, as well as the more common issues associated with protein quantitation in biological samples. Here, we review the workflows that have been used to select peptide targets for food allergen detection. We describe the use and limitations of both in silico-based analyses and experimental methods relying on high-resolution MS. The variation in the way in which target selection is performed highlights a lack of standardization, even around the principles describing what the detection method should achieve. A lack of focus on the food matrixes to which the method will be applied is also apparent during the peptide target selection process. It is hoped that highlighting some of these issues will assist in the generation of MS-based allergen detection methods that will encourage uptake and use by the analytical community at large.

Undeclared food allergens represent a substantial food safety concern for food manufacturers, regulators, and allergic consumers. In order to assess allergen control plans, confirm regulatory compliance, and conduct quantitative risk assessments, methods to reliably detect and quantify food allergens are required. Immunoassay-based methods (including ELISA and lateral-flow devices) are currently the most widely used detection methods for food allergens. PCR is also used in some situations, depending on the allergenic food target and the food matrix in question. Although immunoassays are useful in a number of instances, they can also have difficulties providing accurate quantification and detection in certain food matrixes or following certain types of food processing (1–3).

For the major allergenic foods, the protein fraction of the food is responsible for reactions in allergic individuals. For most allergenic foods, more than one individual protein is responsible for reactions across different individuals, and one allergic individual can be reactive against more than one protein from a food. Due to this heterogeneity in individual reactivity patterns, the presence of any protein from the allergenic source is generally considered to be a potential hazard. Because proteins are the food component of concern, proteomics techniques, and, more specifically, protein MS methods, can be used to analyze for the presence of food allergens. In principle, well-established proteomics techniques from other fields can be applied to food allergen analysis. In practice, however, detection and quantification of food allergens by MS can present some unique challenges.

Bottom-Up Proteomics Techniques

Due to the complexity of intact protein spectra and challenges associated with protein dissociation, a wide majority of protein MS studies conducted across different disciplines and applications have followed bottom-up proteomics workflows. Bottom-up proteomics techniques have been thoroughly reviewed elsewhere (4, 5). Briefly, bottom-up experiments analyze peptides derived from proteins to infer the identity, quantity, and characteristics of the parent protein. The peptides analyzed by MS are typically produced through the use of proteolytic enzymes, most frequently trypsin, due to its predictable cleavage specificity and production of peptides with favorable ionization properties (4). Proteomics techniques can further be categorized as either discovery or targeted methods.

Discovery proteomics methods collect MS data in a blinded fashion with respect to which peptides or proteins will be observed or identified. Most often, the identification of peptides, and subsequently proteins, is conducted through the use of one or more search algorithms that compare experimental mass spectra to theoretical spectra generated in silico from protein sequence databases (6–12). Depending on the instrumentation and the purpose of a given experiment, both data-dependent acquisition and data-independent acquisition strategies can be implemented for discovery proteomics methods (4). Although discovery methods provide a global view of a particular protein sample, they generally have lower sensitivity and less reliable quantification.

Unlike discovery methods, targeted proteomics methods focus on the acquisition of spectra for predetermined sets of peptides or proteins. One of the most commonly used targeted proteomics techniques is selected-reaction monitoring (SRM). In an SRM

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experiment, sets of specific precursor and product ions, collectively known as transitions, are monitored to indicate the presence of a given target peptide (13). The measured signal intensity of the transition fragment ion can also be used for quantification, and with the use of isotopically labeled internal standard peptides, SRM methods can deliver absolute quantification data (14, 15). SRM experiments are typically conducted on triple quadrupole (QqQ) instruments, with precursor ion selection occurring in Q1, fragmentation taking place in Q2, and product ion selection occurring in Q3.

Although SRM assays require the preselection of both precursor and product ions, a newer targeted proteomics strategy known as parallel-reaction monitoring (PRM) only requires the precursor ion to be predetermined (16, 17). Unlike SRM experiments, PRM methods use quadrupole Orbitrap or quadrupole time-of-flight (TOF) instruments. In PRM assays, preselected peptide precursors ions are selected by the quadrupole, each ion is fragmented, and the corresponding full product ion spectra are analyzed in the Orbitrap or TOF mass analyzer. Although QqQ instruments generally have more efficient duty cycles than TOF or Orbitrap instruments, the high-resolution capabilities of these mass spectrometers can help distinguish product ion interferences in complex mixtures (16–18). The acquisition of full product ion spectra can also provide additional confidence in peptide identity. As with SRM assays, PRM methods can be used for relative and absolute peptide quantification.

Because targeted MS methods generally deliver better sensitivity and more accurate quantification, they continue to be the methodology of choice for MS-based detection and quantification of food allergens. As only predetermined peptides or transitions will be observed in these methods, the selection of target peptides is a critical part of method development. Foods, however, are perhaps the most complex commonly analyzed samples in proteomics, considering not only the metaproteomic nature of many food products, but also the diverse range and complexity of processing-induced effects. To a large degree, this means that target peptide selection for food allergen methods cannot be conducted in isolation from other methodological considerations, such as protein extraction and digestion. This review discusses the efforts that researchers and analysts undertake to select high-quality target peptides and generate effective methods.

Considerations for Food Allergen Method Development

In addition to the MS-specific factors affecting method development, which are addressed below, there are a number of overarching considerations that may influence the direction of a target selection process. A general method to detect multiple allergen-derived ingredients in a wide array of foods will likely require different target selection strategies than a method designed to detect a particular allergen-derived ingredient in a specific product. A thorough understanding is required of both the allergenic source to be detected and the matrix in which it will be detected. Although different types of characterization data are applicable for MS and ELISA methods, there are similar considerations for both in terms of the need to assess method suitability for particular allergen-derived ingredients and matrixes.

For MS methods, familiarity with the allergenic food should start with knowledge of how well the proteome has been characterized. Often, the protein sequence database information for foods, including allergenic foods, is rather minimal. For example, as of this review, there are currently a total of 16 protein sequences for cashew (*Anacardium occidentale*) in UniProt. Even in cases in which full genomes and reference proteomes are available, adequate annotation of proteins, isoforms, and proteoforms is lacking. All peptide target selection strategies rely on protein sequence databases in some way or another, making thorough examination of the information that is and is not present in the databases a critical piece of method development. In this way, MS method development differs from ELISA development. Antibody-based methods recognize specific and localized regions of protein and are often dependent on the conformation of their protein targets for recognition. However, the targets of antibody-based methods are seldom identified to any greater degree than the protein on which the target is found. Thus far, all MS methods for allergen detection have been based on known peptide sequences.

Beyond understanding the theoretical proteome of the allergenic food on an informatics level, it may also be important to understand the actual proteome of allergen-derived ingredients that are to be detected, depending on the purpose of the method. Like many foods, allergenic foods are frequently processed to improve safety, functionality, and organoleptic properties. These processes can range from thermal treatments, such as drying, roasting, and pasteurization, to fermentation, protein fractionation, concentration, and isolation. Milk is an allergenic food that can be particularly illustrative of this point. The form of milk most obviously encountered by consumers is fluid milk. Even in this form, milk has already undergone several processes, including homogenization and pasteurization. The food industry, however, commonly produces and uses ingredients derived from fluid milk rather than the fluid milk itself. These ingredients can include fractionated protein products, such as sodium caseinates, whey powders, and whey protein concentrates and isolates. Considering which of these types of allergen-derived ingredients need to be detected in a method becomes important in the target peptide selection process.

In addition to thoroughly understanding the allergenic food ingredients to be detected, consideration of the matrix in which the allergen will be detected is also important. First, in order to avoid false-positives, target peptides selected from the allergenic food must not be present in the background matrix food. Although this type of screening for identical peptides is typically conducted by searching protein sequence databases, proteome-level or even minimal amounts of protein sequence information is not available for the vast majority of background foods. Therefore, identification of peptides present in relevant background foods may need to be conducted empirically. As is the case with allergen-derived ingredients, the types of processing applied to the food matrix should also be taken into consideration, as peptides and/or proteins may be differentially affected by the processing. The use of incurred foods, which have been used for ELISA and PCR allergen method validation, is also valuable in the target selection and method validation processes for MS methods (19).

Bioinformatics/in Silico Target Selection

Bioinformatics approaches to peptide target selection begin with the databases that house protein sequence information, which can include public (e.g., UniProt and National Center for Biotechnology Information) or proprietary databases (20, 21). Specific protein targets with known sequences are selected from these databases. In the case of food allergen detection methods, this typically involves selecting relevant allergenic proteins and/or major food proteins, particularly when clinically relevant allergenic proteins are poorly defined for a given food. Once target protein sequences are chosen, an in silico digestion (most frequently with trypsin) is performed to deliver potential target peptide sequences.

A complete list of theoretical peptides typically is narrowed down to a shorter list of potential target peptides that should perform well using a number of filters or in silico tools. Other authors have reviewed peptide-filtering criteria (e.g., length, propensity for modifications, uniqueness, etc.) that should deliver high-quality target peptides (13, 22, 23). There are also in silico tools to identify peptides prone to missed cleavages and predict high-quality quantitative peptides (24, 25). If an SRM method is being developed, potential transitions must be determined for the selected target peptides. Although this can be done manually, software such as Skyline is frequently used for the in silico prediction of optimal transitions (26).

The benefits of bioinformatics-based target selection include the lack of a need for high-resolution MS instruments (i.e., those with TOF, ion cyclotron resonance, and Orbitrap mass analyzers), which are frequently costly and less commonly available. In addition, the resources and inputs required to conduct in silico analyses are relatively minimal compared with discovery-based target selection strategies. Bioinformatics-only selection strategies also have their drawbacks, however. For many foods, there is little protein sequence information present in the databases, and even when sequences are available, the selection of appropriate protein and peptide sequences can be challenging. For example, in allergenic plant-derived foods, it can be difficult to predict from database information which sequence isoforms or proteoforms will be present or predominant in the food itself. Protein sequence database information also does not incorporate information about how proteins or peptides are affected by processing applied to the ingredient itself or finished food products. In addition to the lack of protein sequence knowledge for the allergenic food in question, there is also a substantial lack of sequence information for other background foods, making in silico determination of peptide uniqueness challenging.

Despite the challenges of bioinformatics-based target selection, some successful food allergen detection methods utilizing primarily in silico target selection have been published (27–33). Several of these methods have been described for the detection of milk proteins in various foods (28, 30, 31). Notably, Lutter et al. (30) used predominantly bioinformatics strategies to select peptide targets for a method to quantify milk proteins in soy-based infant formula, breakfast cereal, baby food, and infant cereal. The depth of existing knowledge about milk protein physiochemical properties, behaviors, and sequences may make them particularly well suited for in silico target selection. In the development of a method to detect milk, egg, peanut, and soy, Planque et al. (32) used a different strategy for target peptide and transition selection that incorporated both in silico and empirical data. These authors first conducted a bioinformatics-based selection of protein sequences from UniProt and performed in silico digestion and transition prediction in Skyline. Instead of using peptide and transition filters, however, the authors then collected SRM data on the large number of theoretical peptides and transitions in the allergenic source foods and in food with high levels of incurred allergens. This combination of bioinformatics selection and SRM data collection allowed the authors to make decisions on peptide targets using relevant empirical evidence without requiring high-resolution instrumentation.

Going forward, bioinformatics-based target selection strategies for food allergen assays could be substantially enhanced by the development of peptide spectral libraries, SRM assay databases, and discovery proteomics data repositories, as is the case for other proteomics applications (34–36). The development of these community informatics resources can reduce the time, expertise, and inputs required to develop targeted methods by providing existing data from multiple laboratories of either discovery or targeted proteomics experiments.

Discovery-Driven Target Selection

For a variety of reasons, many developers of targeted food allergen methods find benefit in beginning the target selection process with discovery proteomics experiments (37–50). Bottom-up discovery proteomics analyses are typically conducted on the allergenic food in question, although specific purified protein(s) or protein fractions from the allergenic food can also be used, depending on the goal of the final targeted method. As in other discovery proteomics applications, tandem MS data from the target food or protein is searched against a relevant protein sequence database to obtain peptide identifications.

Particularly with modern instrumentation, these discovery proteomics workflows result in extensive lists of peptides from the allergenic food, which must be narrowed down to a shorter list of candidate target peptides. A number of different parameters can be used for this process, including empirically derived values, such as peptide abundance (e.g., as determined by the MS1 peak area), MS2 spectral quality (e.g., number of identified fragment ions), and the observation of missed-cleaved or otherwise modified versions of a peptide (46). Method developers have also frequently incorporated additional in silico selection criteria, including peptide specificity (as determined by Basic Local Alignment Search Tool searches against known protein sequences), peptide composition restrictions (e.g., no methionine residues allowed), and peptide length requirements (42, 44, 45, 48, 49). The various types of selection criteria used by individual researchers are often quite variable, are frequently used in an ad hoc fashion, and may require extensive manual data review.

The implementation of discovery proteomics in the peptide selection process can have a number of benefits. As discussed above in reference to bioinformatics-based selection, the knowledge of specific protein and proteoform presence and abundance in a given food is frequently lacking. Conducting discovery proteomics experiments on the allergenic food

source provides confirmation that a given peptide is present and observable in the food itself. Additionally, the acquisition of full product ion spectra, rather than only SRM data, provides enhanced confidence that a given ion is correctly attributed to a particular peptide. Finally, the variability in protein or peptide observation among different types of allergen-derived ingredients can be determined by conducting discovery proteomics on a relevant selection of these products.

Despite the advantages of incorporating discovery proteomics into the target selection process, there are also some notable drawbacks. In particular, unless de novo sequencing is conducted (and, in most cases, it is not), identification of peptides through discovery workflows still requires protein sequences to be known in advance, as nearly all discovery data analysis relies on searches against protein sequence databases. In addition, large-scale discovery experiments often require high-resolution instrumentation, which may not be accessible to all method developers. Also, depending on the food in question, the analysis of discovery proteomics data can be quite complex and time-consuming.

Peptide Selection Considerations

Protein Extraction

The product of the initial extraction of protein from a food matrix represents the entirety of proteins that are available for further analysis and the form in which they are present. This being the case, it may reasonably be stated that extraction is the most important step in method design. There are few predictive tools available to method developers to assist in predicting how a given extraction may work in a given food matrix. However, basic knowledge of how different families of proteins typically extract in aqueous conditions can help, e.g., the prolamins of peanut (Ara h 2, 6, and 7) appear to be more soluble under aqueous conditions than other major proteins in the peanut seed (51). Such basic biochemical knowledge may help identify which proteins are likely to be soluble, extractable, and, therefore, a good source of peptides for method design. However, we cannot say how protein solubility will change from food matrix to food matrix without experimentation. Typically, extraction conditions are assessed to see which yield the greatest amount of protein from a food allergen using total protein detection methods or using semidiscriminatory techniques, such as sodium dodecyl sulfate polyacrylamide gel electrophoresis, allowing the estimation of the amount of a particular protein (52). Different extractions may be performed, with the one yielding the most protein taken for further method development. The usual aim is to obtain an extraction that best represents the protein composition of the allergenic food, usually in the absence of a food matrix. An additional consideration is that extraction conditions that are expensive or difficult to perform on large sample amounts may be identified as optimal. The amount of food extracted is a key consideration in sampling, and larger amounts are preferable to avoid issues with sample heterogeneity (53, 54). In food analysis, we are seldom limited by available sample, and large amounts of sample and, therefore, larger volumes of extraction are preferable.

One key aspect of extraction is the presence or absence of chaotropes that can assist extraction, especially from samples containing aggregated proteins (55). This is not generally the case for antibody-based detection methods, due to the involvement of protein structure in antibody binding. Chaotropes and detergents must typically be removed prior to MS analysis, usually in a dedicated step involving filtration or precipitation (56). The choice of whether or not to include chaotrope(s) in an extraction is largely one of efficiency versus convenience, but their inclusion in an extraction is often recommended as a way to address the diversity of food matrixes and processing encountered (57).

The extraction step of method development is seldom chosen to allow the analysis of particular peptide targets, but nonetheless greatly effects which peptide targets may be subsequently chosen. Although extraction conditions that extract the majority or all proteins from a target allergenic food are most often sought, there is no a priori reason why this should be the case. In principle, the only molecules that are required to be extracted are those that are to be used as target peptides in a final detection method. Selective extractions for analysis are already accepted for many food analyses that target small molecules. AOAC INTERNATIONAL's *Official Method* for pesticide residues, Method **2007.01**, uses an acetonitrile extraction followed by solid-phase extraction, resulting in a heavily enriched set of analytical targets (58). Similarly, many MS-based mycotoxin determinations consider only the effective extraction of the target molecules (59). The clinical measurement of protein and peptide markers in human blood relies on heavy selective enrichment using immunochemical techniques (60). Emphasizing complete extraction of all proteins from an allergenic food source results in extraction methods that require removal of chaotropes often not suitable for the extraction of the large amounts of food required for robust analysis and, in any case, are not guaranteed to work in the diversity of food matrixes against which the method will function. It is, instead, preferable to base MS detection methods on proteins and peptides that are readily extracted under the rapid and low-cost conditions necessary for food analysis.

Digestion Conditions

Hydrolysis of proteins into the peptides used as analytical proxies is likely the most consistent step in allergen detection by MS. With very few exceptions, trypsin (cutting to the C-terminus of lysine and arginine) is used. Furthermore, the conditions (time, buffer, and temperature) used for digestion are usually those provided by the manufacturer of the enzyme or digestion kit. Digestions are usually performed in solution. The efficiency of trypsin cleavage and, therefore, the likelihood of a "missed cleavage" can be, to some extent, predicted based on protein sequence (25), with the usual caveat that the diversity of the food matrix may influence trypsin activity. The possibility that target proteins may be cleaved by proteases in the food matrix is lessened by preceding reduction and alkylation, which will likely destroy the activity of such proteases.

The near-ubiquitous use of trypsin limits the choice of target peptides. In some cases, other enzymes have been used in mixed digestion systems with trypsin. The enzyme Lys-C has similar sequence specificity to trypsin, but differing physicochemical optima, which make it a logical choice to use in addition to trypsin without compromising the use of tryptic peptides (61).

The previous authors also used chymotrypsin to generate peptide targets for gluten detection, although care must be taken to ensure target peptides are generated efficiently and specifically in such cases. Overall digestion efficiency can be improved by addition of more than one proteolytic enzyme with different sequence specificities (62).

Conclusions

The field of targeted proteomics has advanced rapidly over the past decade, and a number of useful principles, tools, and resources for targeted method development are now available. However, researchers developing targeted MS methods for food allergen detection and quantification face a number of challenges that are unique among other proteomics applications, but shared among other approaches (ELISA and PCR) to allergen analysis. The complexity of both allergenic foods and the food matrixes in which detection is sought, combined with the lack of protein sequences and sufficient annotation in databases for these foods, results in a number of obstacles for the prediction of suitable target peptides and transitions. The relative lack of sequence information for commonly consumed foods, allergenic or otherwise, is a notable impediment. Method development workflows that take into consideration relevant food matrixes, processing techniques, and allergen-derived ingredients during the target selection process are likely to be more successful when methods are subsequently validated with incurred foods.

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